THE EFFECT OF THYMOL-B-D-GLUCOPYRANOSIDE ON THE REDUCTION OF *CAMPYLOBACTER* SPECIES IN FOOD-PRODUCING ANIMALS

A Thesis

by

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ABSTRACT

Campylobacter are a leading cause of bacterial derived foodborne illness. Thymol is a natural product that reduces survivability of *Campylobacter in vitro*. Results from animal studies, however, indicate that absorption or degradation within the stomach and small intestine may preclude delivery of thymol to the cecum and large intestine, the main sites of *Campylobacter* colonization. Presently, we compared the anti-*Campylobacter* activity of thymol against that of thymol-β-D-glucopyranoside (β-D-thymol), the latter suspected to be resistant to degradation and absorption in the proximal alimentary tract lacking β-glycosidase activity. When treated with 1 mM thymol, the survivability of *Campylobacter coli* and *jejuni in vitro* was reduced by 3.41 to 6.87 $\log_{10}$ CFU mL^{-1} after 48-h pure culture and after co-culture, respectively. In the presence of a β-glycosidase-expressing *Parabacteroides distasonis.* Conversely, the survivability of *C. coli* and *C. jejuni* was reduced by 3.72 and 4.30 $\log_{10}$ CFU mL^{-1}, respectively, in co-cultures treated with β-D-thymol, but not in pure cultures similarly treated. When tested in mixed cultures of porcine or bovine fecal microbes possessing endogenous β-glycosidase, *C. coli* and *C. jejuni* survivability was reduced by 3.26 and 2.50 $\log_{10}$ CFU mL^{-1}, respectively, whether treated with thymol or β-D-thymol. In mixed populations of avian crop and cecal microbes, *C. jejuni* survivability was reduced 1.41 to 2.32 $\log_{10}$ CFU mL^{-1} whether treated with thymol or β-D-thymol. Thymol and β-D-thymol inhibited ammonia accumulation in mixed populations of porcine and mixed bovine fecal microbes which is consistent with free thymol's purported role as a deaminase

inhibitor. Conversely, thymol and β-D-thymol did not affect ammonia accumulation in mixed populations of avian gut microbes implicating population specific effects of these compounds. β-D-thymol, but not thymol, reduced accumulation of fermentation acids indicating the conjugate inhibited fermentation which may limit its application to the last meal or last few meals before harvest. Oral administration of 150 μmol β-D-thymol reduced *C. jejuni* in avian crop, but not in cecal contents; treatment with thymol was ineffective. These results indicate that β-D-thymol, or similar β-glycosides, may be a suitable candidate to escape absorption and degradation within the proximal alimentary and retain its anti-*Campylobacter* properties. Further research is needed to reduce such technology to practice.

DEDICATION

To my parents, John H. Epps (deceased) and Rosie L. Epps, who have faithfully encouraged and supported me through all my endeavors in life.

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NOMENCLATURE

ATP	Adenosine Triphosphate
β–D-Thymol	Thymol-β-D-Glucopyranoside
CFU	Colony Forming Units
GBS	Guillian Barre Syndrome
MFS	Miller Fischer Syndrome

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Introduction

Campylobacter spp. are a leading cause of bacterial associated human foodborne illness in the United States. Campylobacteriosis is estimated to affect over 1 million people every year and to cause 100 deaths. The cost of human illness is $1.3 to $6.2 billion annually (1, 2). *Campylobacter* can also cause post-infection complications associated with acquired immune-mediated neuropathies such as Guillian Barre Syndrome or Miller Fischer Syndrome (3, 4). Most human *Campylobacter* infections are caused by *Campylobacter jejuni*; however 4% of clinically confirmed cases in one study were attributed to *Campylobacter coli* (5). Usually cattle and poultry are colonized with *Campylobacter jejuni* and pigs are colonized with *Campylobacter coli*. However, considerable numbers of pigs can be colonized with *Campylobacter jejuni* (6, 7). *Campylobacter* are also recognized as reservoirs for antimicrobial resistance genes that potentially can be exchanged between other pathogenic and commensal bacteria (8-11). Poultry, swine and cattle can harbor these pathogens within their digestive tracts and risk contaminating the carcass when it is presented for processing.

Unlike many other gut bacteria, *Campylobacter* are limited in their ability to conserve energy for growth and maintenance via fermentation of carbohydrates. These bacteria lack 6-phophofructokinase, which is a key enzyme in energy metabolism (12). *Campylobacter* can conserve energy via respiration, oxidizing hydrogen and formate for

the reduction of electron acceptors such as fumarate, nitrate, sulfites and if at low concentrations, oxygen, to generate proton motive force transport phosphorylization (13-17). It is unclear how much growth can be supported by this process, since concentrations of these electron acceptors are typically low within the gut environments.

Campylobacter have the capability to catabolize amino acids such as aspartate, alanine, glutamate, glutamine, methionine and serine at a substrate-level to store energy (18-20). Intermediate and end products of amino acid catabolism by *Campylobacter* include acetate, formate, fumarate, lactate, pyruvate and succinate, thus indicating that the involvement of mixed acid fermentation and reductive metabolism of the tricarboxcylic acid pathway (21, 22).

The purported deaminase inhibitor thymol, an extract of thyme, is a natural product that markedly reduces survivability of *Campylobacter in vitro*. Thymol disintegrates the outer membrane of Gram-negative bacteria releasing lipopolysaccharides and increasing the permeability of the cytoplasmic membrane to ATP. ATPases are located in the cytoplasmic membrane and are bordered by lipid molecules. There are two suggested mechanisms where cyclic hydrocarbons act: 1) hydrocarbon molecules accumulate in the lipid bilayer and distort the lipid-protein interaction; 2) direct interaction exists between the lipophilic compounds the hydrophobic parts of the protein (23-25). Although there is still some debate about the mechanism of action of thymol, previous studies have shown thymol to be effective in reducing microbial growth (24, 26, 27). However, results from *in vivo* studies revealed that thymol is absorbed in the stomach and small intestine, thereby preventing delivery

of efficacious amounts of thymol to the cecum and large intestine where *Campylobacter* reside (28).

This study proposes that attaching thymol to glucose to form a thymol-β-D-glucopyranoside that will make it a suitable product capable of bypassing the stomach and small intestine. The enzyme β-D-glucosidase, which is present in bacteria, molds, and yeast, retains catalytic activity over the pH range 2.0-9.5. This enzyme exhibits D-galactosidase activity that should act as a hydrolysis mechanism that can release thymol from the β-D-glucopyranoside once delivered to the cecum and large intestine.

Reduction of food borne pathogens prior to slaughter could reduce human exposure to pathogens and thus reduce the numbers of human food borne illnesses. The use of natural essential oils, such as thymol and thymol derivatives, in place of traditional antibiotics can lead to viable treatments that will lessen dependence on antibiotics and reduce the occurrence of food borne illness in humans. The overall project goal is to develop and produce a practical, cost effective, and easy way to implement pre-harvest strategy to reduce the incidence and concentration of *Campylobacter* in the gut of food-producing animals immediately prior to processing.

Literature Review

Campylobacter species

Campylobacter spp. are Gram-negative spiral non-spore forming rods that form spherical or coccoid bodies in older cultures. They are between 0.2 to 0.9 microns wide and 0.5 to 5 microns long, are motile and usually move with a polar unsheathed

flagellum at one or both ends, and are microaerobic with a respiratory-type metabolism, although there are some that grow aerobically or anaerobically (29).

The *Campylobacter jejuni* genome is a relatively small genome; 1.6-1.7 Mbp of adenine and thymine-rich DNA content and the guanine and cytosine content ranges from 29 to 47% (30-32). *Campylobacter* spp. is one of the leading causes of bacterial-associated human foodborne illness in the United States. The rate of *Campylobacter* infections is increasing worldwide, exceeding shigellosis and sometimes exceeding salmonellosis (33, 34).

The infectious diseases caused by members of the bacterial genus *Campylobacter* are called campylobacteriosis (34). Currently *Campylobacter jejuni* (*C. jejuni*) and *Campylobacter coli* (*C. coli*) are considered to be the most important enteropathogens among *Campylobacter* spp. (35).

Campylobacter **history**

From a historical standpoint, the first report of *Campylobacter* species is believed to have been made in 1886 by Theodore Escherich, who observed and described a non-culturable spiral shaped bacteria, which he found in the colon of children with an enteric disease called, "cholera infantum" (30, 36-38). *Campylobacter* was identified in 1913 by two British veterinarians, J. McFadyean, and S. Stockman. McFadyean and Stockman reported the presence of "large numbers of a peculiar organisms" in the uterine mucus of a pregnant sheep (36, 39-41). In 1927 a group of vibrio-like bacteria was found in the feces of cattle with diarrhea. They were described by Theobold Smith and Marion Orcutt (35, 42, 43). In 1931, S.F. Jones and coworkers

showed a relationship between the microaerophillic vibrios and bovine dysentery, and the organism was eventually called *Vibrio jejuni*. The first well documented incident of *Campylobacter* infection took place in Illinois in 1938. The case involved a milk-borne outbreak of diarrhea that affected 355 inmates in two adjacent state institutions (41, 44). In 1944, Michael P. Doyle isolated another vibrio from the feces of pigs with diarrhea and classified it as *Vibrio coli* (35, 45). Because of their low DNA base (Low Guanine and Cytosine) composition, non-fermentative metabolism and their microaerophillic growth requirements the genus *Campylobacter* was proposed by Seabald and Vernon in 1963, distinguishing them from the *Vibrio* spp. (36, 46). There are two subspecies recognized within *C. jejuni*, *C. jejuni* subspecies *jejuni* and *C. jejuni* subspecies *doylei*. Strains of *doylei* differ from *jejuni* biochemically. *Campylobacter coli* and *C. jejuni* differ biochemically in their ability to hydrolyze hippurate. *Campylobacter coli* cannot hydrolyze hippurate and there are some *C. jejuni* subspecies that are hippurate negative (35).

Campylobacter **metabolism**

Campylobacter genus members are limited in their ability to conserve energy for growth via fermentation of carbohydrates due to the absence of two key components of the Embden-Meyerhof-Parnas pathway: The first component is the phosphoenolpyruvate dependent phosphotransferase system, which transports and phosphorylates sugars simultaneously, and the second component is phosphofructokinase, which catalyzes the conversion of fructose-6-phosphate to fructose 1,6-biphosphate (12, 35). *Campylobacter* can conserve energy via respiration, oxidizing hydrogen and formate for the reduction of

the electron acceptors (fumarate, nitrate, sulfites) and, if at low concentrations, oxygen, to generate proton motive force for electron transport phosphorylation (12-16). *Campylobacter jejuni* relies on the use of the amino acids and the citric acid cycle intermediates as carbon sources (47). There are some *C. jejuni* subspecies *doylei* which contain some enzymes from the Enter-Deudoroff pathway which convert glucose-6-phosphate to glyceraldehyde-3-phosphate utilizing gluconate-6-phosphate instead of a fructose-6-phosphate intermediate. This pathway eliminates the need for phosphofructokinase (35). It has generally been assumed that *C. jejuni* were found to contain genomic islands (cj0480c-cj0490) that are up regulated in the presence of L-fucose and mucin obtained from the host during colonization in the intestine (48). The current knowledge of *C. jejuni in vivo* metabolism is based on amino acid utilization to support the growth and establishment of colonization in the host intestines. *Campylobacter jejuni* metabolic diversity is evidenced by its differential carbon utilization (49-51).

Campylobacter antimicrobial resistance

Antibiotic resistance is primarily due to genetic mutations in bacteria causing resistance to the drug and allowing for survival of the pathogen. Many of these resistant genes are located on plasmids, allowing for easy transfer. A 2011 review published in Frontiers in Microbiology hypothesizes that the unregulated use of antimicrobial agents in food animal production has led to the emergence and spread of antibiotic resistance among *Campylobacter* spp. The evidence of this is strongly supported (36). Antibiotics have been used for decades in food production animals to control, prevent, and treat

infections and to enhance growth (36, 52-54). This usage has caused an increased resistance to multiple antibiotics by members of *Campylobacter* spp. in food production animals and environments (36, 55). Food production animals are considered to be the primary source of *Campylobacter* infections in humans, thus the development of antimicrobial resistance in *Campylobacter* spp. is a serious threat to human health (36).

Worldwide, there has been a rapid increase in the proportion of *Campylobacter* strains resistant to antimicrobial agents (56-59). *Campylobacter jejuni* and *C. coli* are almost universally resistant to penicillians, cephalosporins (with exception of a few 3rd generation cephalosporins), trimethoprem, sulfamethoxazole, rifampicin and vancomycin (35). *Campylobacter jejuni* and *C. coli* cause a broad range of illnesses in humans. Fevers, abdominal cramping, and diarrhea, with or without blood in stools, are distinct features of an uncomplicated illness that could last from a few days up to a week (35, 60). The prognosis is that most patients infected with *Campylobacter* spp. will recover without specific treatments other than replacement of fluid and electrolytes, despite the fact that in more severe cases generally antibiotics such as macrolides, tetracycline, and fluroquinolones are administered (61). It is believed that increasing resistance to fluroquinolones, tetracycline, and erythromycin by *C. jejuni* and *C. coli* might compromise the effectiveness of these treatments (8, 36, 62-64).

Human estimates

In 2005, the Centers for Disease Control and Prevention (CDC) estimated that the number of campylobacteriosis cases in the United States was about 1 million per year. In 2005, *Campylobacter* was the second leading cause of laboratory confirmed

cases of foodborne illness in the United States, with an estimated 12.72 cases per 100,000 people, for a total of 5,655 cases (65). In 2009, the Foodborne Disease Active Surveillance Network (FoodNet) of the CDC estimated that the number of infections by *Campylobacter* was a total of 6,033, or 13.02 per 100,000 people (36). In the last five years the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC) reported that campylobacteriosis has become the most often reported zoonosis in the European union followed by salmonellosis and yersinosis (36, 66, 67). More than 200,000 confirmed cases of campylobacteriosis were reported in 24 of the member states of the European Union at a rate of 45.2 cases per 100,000 people. In 2010 New Zealand reported the highest national campylobacteriosis rate, which peaked in May 2006 at 400 per 100,000 population (36).

In general, developing countries do not have national surveillance programs for campylobacteriosis; thus, there are no case incidence values in terms of population density (33). Most of the estimates of incidence are done by laboratories where the surveillance is based on pathogens responsible for diarrhea.

Campylobacter isolation rates range from 5 to 20% in developing countries (33, 68). Most of the data are collected by the World Health Organization (WHO).The WHO and the Canadian Public Health Service have provided financial support to developing countries for epidemiologic studies (33, 69). There is a large disparity in the incidence of campylobacteriosis in developing countries versus that of developed countries. High numbers of children in developing countries are affected by *Campylobacter*. Community based studies in developing countries have estimated that 60,000 per 100,000 children <

5 years of age (33, 68, 70) are infected; whereas, in developed countries 300 per 100,000 are affected (33, 71). Estimates for the general population in developing and developed countries are relatively similar at approximately 90 per 100,000 affected by campylobacteriosis (33, 69, 71). This result suggests that campylobacteriosis is a pediatric disease in developing countries. In developed countries, more than 90% of human campylobacteriosis cases occur during the summer because of undercooked meats from outdoor cooking facilities. People of all ages are affected, but particularly children less than 4 years of age and young adults 15-44 years of age (2, 41).

The infectious dose of *Campylobacter jejuni* for humans is estimated to be low; between 500-800 organisms. The dosage was estimated in 1981 as a result of a human experiment in which a British medical doctor, D.A. Robinson, swallowed 500 organisms of known serotype in 180 ml of pasteurized milk. The findings from this self-inflicted experiment, which reportedly satisfied the criteria of Koch's Postulates and the findings of additional human experiments, verified a dosage and a mechanism of *C. jejuni* human infection (35, 72). In industrialized countries, clinical signs of campylobacteriosis are characterized by acute self-limited gastrointestinal illness with abdominal cramps, diarrhea and fever. The incubation period is from 2 to 7 days (41). In severe cases of *C. jejuni* infection, individuals may exhibit fever, abdominal cramps and diarrhea that contain blood and leukocytes, or they may develop post infection complications associated with Guillian-Barre Syndrome (GBS) or Miller Fischer Syndrome (MFS). MFS is a subform of GBS characterized by areflexia, ataxia and opthalmoplegia (35, 73).

Campylobacter pathogenesis and disease

The exact sequential steps of *C. jejuni* infection to *C. jejuni* mediated enteritis are unknown. What is known are the requirements for *C. jejuni* virulence: 1) motility, 2) drug resistance, 3) host cell adherence, 4) host cell invasion, 5) alteration of the host cell signaling pathways, 6) induction of host cell death, 7) evasion of the host 8) immune system defenses, and 9) acquisition of iron which serves as a micronutrient for growth and works as a catalyst for hydroxyl radical formation (74, 75). It is also known that *C. jejuni* secretes proteins that contribute to the ability of the bacterium to invade the host epithelial cells. A suggested model for *C. jejuni* pathogenesis shows the first step as the consumption of *C. jejuni* contaminated food or food products. Survival in the host environment depends on several adaptive responses including adherence, protein secretion, invasion and replication.

The biochemical effects on cellular events are as follows: 1) cytoskeletal rearrangement, 2) host cell death, 3) tight junction disruption and cytokine induction that leads to loss of epithelial cell function, 4) a compromised barrier and absorptive functions, and 5) tissue destruction and disease manifestation. Adherence can also lead to an early inflammatory response that causes stimulation of innate immune functions in the following sequence; 1) an influx of fluid, 2) complement cell activation, 3) recruitment of phagocytes that can lead to *C. jejuni* lysis or death, 4) clearing of the infection or the presentation of antigens, 5) a humoral response and, 6) a clearing of the infection. In the other direction, instead of *C. jejuni* lysis or death, the inflammatory response could lead to tissue destruction and disease manifestation (74).

There is evidence that *C. jejuni* infections commonly precede GBS conditions that trigger antibodies which cross react with gangliosides and damage peripheral nerve tissue. In patients who exhibit a *C. jejuni* infection preceding GBS, GBS is considered to be a disease mediated case of molecular mimicry (35). GBS is a rare autoimmune disease characterized by the demyelination of motor and sensory nerves or deterioration of axonal nerves of the peripheral nervous system. This nerve damage can lead to muscle weakness, paralysis, and death. It is reported that GBS "has become the most frequent cause of acute flaccid paralysis since the near elimination of poliomyelitis in the world" (4, 76-78).

Studies have established GBS as a mechanism of molecular mimicry based on Koch's and Witebsky's postulates. They are as follows: 1) the establishment of an epidemiological association between the infectious agent and the autoimmune disease, 2) the identification of T cells or antibodies directed against the patient's target antigens, 3) the identification of microbial mimics of the target antigen, and 4) reproduction of the disease in an animal model. Autoantibodies are the pathogenic component that triggers GBS (77).

There are two major sub-forms of GBS that affect the peripheral nervous system. The major sub-forms of GBS are: 1) acute inflammatory demyelinating polyneuropathy (AIDP) characterized by the demyelination of peripheral nerves, 2) acute motor axonal neuropathy (AMAN) characterized by degeneration of axonal components of peripheral nerves, and 3) Miller Fischer syndrome (MFS) characterized by areflexia, ataxia, and ophthalmoplegia. Studies have indicated that *C. jejuni* infection precedes GBS in 20 to

50% of cases in Europe, North and South America, Japan, and Austraila. The percentage is suspected to be higher in developing countries. Since *Campylobacter* infections occur far more frequently than GBS, neither the characteristics of the host nor the strain of *Campylobacter* species are known determinants of which persons with *Campylobacter* infection contract GBS. Guillian Barre Syndrome usually develops 1 to 3 weeks after a *C. jejuni* infection. Studies in Japan have indicated that the risk of developing GBS may be higher after infection with *C. jejuni* type O:19 (4, 35, 76).

Pathogenic reservoirs and vehicles

Reservoirs are any living or nonliving thing (person, animal, plant, soil, or substance) in which an infectious agent multiplies and develops. Infectious agent reservoirs typically harbor the infectious agent without injury and serve as vehicles that can transmit the infection. The infectious agent depends on the reservoir for its survival. The infectious substance is transmitted from the reservoir to a human or another susceptible host (79). Reservoirs for *C. jejuni* and *C. coli* are as follows: 1) cattle, 2) poultry, 3) pigs, 4) sheep, 5) dogs, 6) birds, 7) apes, 8) rodents, 9) insects, 10) humans, 11) water, and 12) food items (raw milk, pork, beef, lamb, and seafood) (80). *Campylobacter jejuni* and *C. coli* occur mostly as commensal organisms.

Vehicles are modes of horizontal and vertical transmission of infection. Horizontal transmission is defined as transmission of an infectious agent from an infected source to a susceptible contemporary, and vertical transmission is defined as transmission from one generation to the next. Examples of vehicles are: 1) contact with undercooked/raw poultry or meat, 2) contaminated shellfish, 3) unpasteurized raw milk

or dairy products, 4) contaminated and inadequately treated drinking water, and (5) contact with animals, especially young animals (puppies) with diarrhea (58).Transmission routes are: fecal-oral, person-to-person, sexual contact, and direct person-to-person contact which is a rare. There is also speculation that *Campylobacter*, when stressed, enter a viable but non-culturable state characterized by an uptake of amino acids and maintenance of an intact outer membrane (spore like state). In this state organisms can be transmitted to animals. Vehicles and reservoirs are mostly synonymous modes of *C. jejuni* and *C. coli* infection transmission. Even though all reservoirs can act as vehicles, not all vehicles can act as reservoirs. The distinction being that reservoirs allow for the growth and multiplication of enteropathogens through a commensal relationship, whereas, vehicles only allow for transmission of the pathogen.

Incidence of *Campylobacter* infection

From animal farm production to the commercial production of food commodities there are numerous possibilities for transmission of *Campylobacter* infection through cross-contamination. *Campylobacter* infection from consumption of poultry, beef, and pork products is the leading cause of human foodborne illness. Poultry is estimated to account for 50-70% of human *Campylobacter* infection. Live poultry includes broilers, laying hens, turkeys, ducks, and ostriches (35). Studies have indicated that the prevalence of *Campylobacter* colonization in cattle is 0-80% and 20% in sheep. The same study reported that pigs were more contaminated than cattle or sheep (81).

Poultry and poultry products are considered to be the largest contributor of human *Campylobacter* infection. *Campylobacter jejuni* mainly colonizes poultry and is

found predominantly in the cecum and colon, but it can also be found in the crops. Horrocks et al. believe that due to the higher metabolic temperatures of the poultry species they are possibly predisposed to become prominent reservoirs for the thermotolerant *C. jejuni* (82).

Campylobacter transmission is prevalent during preharvest conditions. Colonization in broiler chicks has been demonstrated to be a risk factor in horizontal transmission of *C. jejuni* infection. Broiler chick colonization is estimated to take place no sooner than seven days of age (81, 83). Other studies have found that broiler chick colonization takes from zero to three weeks. Colonization of free-ranging chickens is estimated to take place from 0-8 days (82, 84-86). Although young animals are very susceptible to colonization by *C, jejuni*, older broiler chicks closer to processing age have a higher percentage of *Campylobacter* colonization. Despite horizontal transmission of the flock taking place rapidly, colonization of the flock can take up to several weeks (82). Studies have estimated that up to 98% in the United States and 60% to 80% in Europe of retail chicken meat is contaminated with *C. jejuni*. It was found that the skin and giblets have particularly high concentrations of *Campylobacter* contamination.

Despite the consistent implication that *Campylobacter* colonization of poultry and poultry products is the major contributor to human foodborne illness, cattle and other swine frequently carry *C. jejuni and C. coli*. It is probable that cattle carcasses are contaminated during processing either directly or indirectly. None the less, the cases of

human foodborne illness as a result of *Campylobacter* contamination in bovine products (unpasteurized milk and meat) is a legitimate concern (87, 88).

Multiple studies with cattle have shown that *Campylobacter* preferentially colonize the lower gastrointestinal tract as opposed to the upper gastrointestinal tract (the 1st stomach) where there is a lower pH environment in the rumen. The gallbladder, liver and bile have all been shown to harbor moderately high percentages of *Campylobacter*. In recent studies, 33% and 21.8% respectively of samples tested positively for *Campylobacter* in the gallbladder, its mucosal tissue and bile (82). Unpasteurized bovine milk and milk products are common vehicles for *Campylobacter* foodborne disease transmission. In a study with cattle from a dairy farm 12% of raw milk samples were found to be contaminated with *C. jejuni* possibly as a result of contact with bovine feces, contaminated water or direct contamination as a result of mastitis (89).

The incidence of *Campylobacter* is reported to be lower for forage fed (primarily dairy cattle) than that of feedlot cattle. The possible contributors to the high incidence of *Campylobacter* in feedlot cattle are 1) increased stocking densities, 2) constant contact with feces from other animals, and 3) the high frequency of shared access to community feeding and water troughs (82, 90). These studies found that there was a higher prevalence of *Campylobacter* in feedlot cattle (68%) compared to that of adult cattle from the pasture (7.3%). These numbers remained relatively the same whether tests were conducted before or after transport to processing. These results suggest that confinement may promote increased carriage and horizontal transmission of *Campylobacter*. Further

swab tests on the hides of these cattle yielded the same results. The feedlot cattle had significantly more contaminated hides.

The numbers of swine that are colonized by *Campylobacter* are more comparable to cattle than to those of poultry. *Campylobacter coli,* which normally inhabit pigs intestine, are found on pork products. Swine are predominantly colonized with *C. coli* albeit less frequently they are colonized with *C. jejuni* (91). Young et.al (92) demonstrates that despite the prevalence of *C. coli* in swine, a high prevalence of *C. jejuni* enteric colonization (cecal or rectal contents) has been observed in gilts, sows, and weaned piglets. Studies conducted by Jensen et al. (82, 93) investigated *C. jejuni* versus *C. coli* colonization of outdoor organically-reared pigs to monitor possible shifts from *C. coli* to *C. jejuni* intestinal colonization. There have also been research studies that demonstrate the possible co-existence of *C. coli* and *C. jejuni* in pigs with *C. jejuni* being present in lower numbers (93, 94). Throughout all trials C. *jejuni* was never found to be more prevalent than *C. coli*. The life of swine raised for food production starts at a farrowing barn where they are born. After three weeks they are moved to a nursery where they will stay until they reach 50 lbs (6 weeks). Next they are moved to a finishing unit where they will stay until they reach market growth rate (220-250 lbs). In the finishing unit swine are held 25 per pen. Environmental conditions play an important role in *Campylobacter* transmission the same as cattle and poultry. There are increased possibilities for *Campylobacter* infection and disease transmission in finishing units and breeding farms (92). There are distinct differences in the prevalence and occurrence of

Campylobacter colonization and infection of pigs raised in organic outdoor production systems than those raised in conventional farm systems (95).

Thymol antimicrobial activity

Thymol (C_{10} H_{14} O), also known as 2-isopropyl-5-methylphenol, it is a natural product (monoterpene phenol) found in the essential oil extracted from *Thymus* and *Origanum* plants (Thyme and Oregano). Thymol was discovered by von Casper Neuman in 1719 and it was chemically synthesized and named by M. Lallemand in 1842. Chemically thymol is slightly soluble in water and extremely soluble in alcohols and other organic solvents (96).

Historically essential oils from spices have been used as fragrances, flavoring, and preservatives since ancient times. Thymol and its chemical isomer carvacol were used by the ancient Egyptians in the preservation of mummies (24, 97). The ancient Greeks used thyme oil for aromatic purposes such as incense that were burned in the sacred temples. Thyme was also a symbol of courage to the Greeks. As early as the 16th century thyme oil was used for its antiseptic properties. Thymol is currently used in conjunction with chlorhexidine as a mouthwash (98).

Thymol is an essential oil that belongs to a group of compounds known as biocides, and they are common protection mechanisms used against environmental stressors (herbivores, fungi, and viruses) by most plants. The low frequency of infectious diseases in plants suggests that essential oils would be effective bactericidal agents (99).Thymol has bactericidal and fungicidal properties and has also demonstrated antimutagenic effects in combination with other essential oils. The phenolic components

are primarily responsible for the bactericidal properties of essential oils such as thymol and carvacol (24, 100). Thymols' active components, are generally recognized as being safe for human consumption in the United States, have caused researchers to examine its potential to improve production health in food-producing animals. Thymol has demonstrated bactericidal activity against Gram-negative and Gram–positive bacteria. Research has demonstrated that Gram-negative organisms are slightly less susceptible to essential oils such as thymol than Gram-positive (24).

Thymol's mechanism of action is hypothesized to be disruption of the Gram-negative bacterial cell membrane integrity, which leads to increased permeability to ATPases proteins (98). Cyclic hydrocarbons are lipophillic and therefore are prone to accumulate in lipids. There are two suggested mechanisms where cyclic hydrocarbons act: (1) accumulation of lipophillic hydrocarbon molecules in the lipid bilayer and distortion of the lipid-protein interaction and (2) direct interaction of the lipophillic parts of the protein (23-25). There is some debate about the specific mechanism of thymols' action on *Campylobacter*. Previous *in vitro* studies have demonstrated that thymol markedly reduces the concentration of *Campylobacter* spp. (24, 26, 27). However *in vivo* studies have shown that thymol is absorbed in the stomach and small intestine preventing efficacious amounts from being delivered to the cecum and large intestine (28).

These *in vivo* studies indicate the need for a suitable bypass mechanism capable of delivery of efficacious amounts of thymol to the cecum and large intestine. Attaching a β-D-glucopyranoside to thymol should make it a suitable product capable of bypassing the stomach and small intestine.

CHAPTER II

ACTIVATION OF THYMOL B-D-GLUCOPYRANOSIDE BY BACTERIAL-EXPRESSED B-GLYCOSIDASE

Introduction

Campylobacter spp. are a leading cause of bacterial derived foodborne illness worldwide. *Campylobacter* can colonize the digestive tracts of food-producing animals at a high prevalence. Infections caused by *Campylobacter* originating from food-producing animals are often resistant to a range of antibiotic agents used in food animals and humans. It is possible that the resistant *Campylobacter* and the resistant genes they carry can be transferred to humans by consumption of food products contaminated with the pathogen (99). This could lead to colonization of the human intestinal tract with the resistant *Campylobacter* genes which could change the commensal bacterial environment thereby risking the loss of effectiveness of antibiotics used by humans. *Campylobacter* spp. colonization of humans can also cause post infection complications associated with Guillian Barre Syndrome or Miller Fischer Syndrome (3, 4). Few strategies are available to prevent infection and carriage of contamination by carcasses at the processing plant and fewer yet are available to prevent infection and carriage of *Campylobacter* in animals on the farm (82). Consequently, the food animal industry is continually looking for new strategies capable of reducing the carriage of pathogens on the farm and during processing. Thymol (2-isopropyl-5-methylphenol) is a natural essential oil that markedly inhibits the survivability of *Campylobacter* in pure and mixed

culture in vitro (1). However, *in vivo* studies have demonstrated that thymol is extensively absorbed or degraded within the stomach and small intestine thereby precluding delivery of this compound to the cecum and large intestine where *Campylobacter* primarily reside (101). It is reasonable to hypothesize, however, that thymol conjugates bound to glucose via a β-glycosidic bond may make the bound form of thymol 1) more resistant to absorption and 2) more resistant to degradation within the proximal alimentary tract.

Tests of the first hypothesis have demonstrated that unlike free thymol, thymol-β-D-glucopyranoside is not appreciably absorbed or degraded in everted stomach or small intestinal segments (102). Regarding the second hypothesis, the β-glycoside should be nearly undegradable in the stomach and proximal small intestine of monogastrics because higher animals do not produce the β-glycosidase enzyme required to hydrolyze the β-glycoside. For instance, the lack of β-glycosidase enzymes in monogastrics is the reason why they cannot use cellulose, which is a polymer composed of repeating β-glycosides. Microbes do express β-glycosidase activity, however, and thus the hypothesis predicts that when the β-glycosides of thymol reach microbial activity in the lower gut there should be sufficient microbial β-glycosidase activity to liberate thymol from the glycoside there by allowing it to be active. Accordingly, the objective of this research project was to compare the bactericidal activity of free thymol and the conjugated form, thymol-β-D-glucopyranoside, on *Campylobacter jejuni* and *Campylobacter coli* during pure culture and during co-culture with a β-glycosidase

expressing gut bacterium, *Parabacteroides distasonis*, as well as during mixed culture with populations of swine, bovine and avian gut bacteria.

Materials and Methods

Microbe sources

The present study used *Campylobacter coli*, *Campylobacter jejuni*, and *Parabacteroides distasonis*. *Campylobacter coli* was isolated from a dairy cow (103) and *Campylobacter jejuni* was isolated from a broiler processing facility (104). The *Campylobacter* strains were stored during long term maintenance in CryoCare™ Bacterial Preservers (Key Scientific Products, Round Rock, TX, USA) according to manufacturer's instructions. Each strain was resuscitated for each experiment via initial 24 h culture (39°C) in non antibiotic-supplemented Bolton broth (Oxoid Ltd, Basingstoke, Hampshire, UK) and subsequent plating on Campy Cefex agar prepared and used as described by (105). Isolated colonies were propagated after 48 h incubation at 42º C, were picked and inoculated into Bolton broth supplemented with 0.33 µg of cefoperazone and 200 µg of cyclohexamide per mL, which after 24 h incubation (39°C) served as the stock culture for experiments described below. The *Parabacteroides distasonis* isolate, formerly classified as *Bacteroides distasonis* (106), was obtained from porcine cecal contents grown in continuous flow culture (107).

Tests of thymol and thymol-β-D-glucopyranoside against *Campylobacter* in pure or co-culture with β-glycosidase expressing gut bacterium, *Parabacteroides distasonis*

The effects of thymol and β-D-thymol were tested on *C.coli* and *C.jejuni*, during both pure culture and co-culture with *P. distasonis*, were tested in two separate experiments conducted on separate days. Cultures were conducted in antibiotic-free Bolton broth that had been prepared anaerobically by boiling and then cooling on ice while under continuous stream of 100% N_2 gas. Once cooled, 5% (v/v) laked horse blood (Lampire Biological Laboratories Pipersville, PA, USA) was added and 10-mL volumes of media were aseptically distributed to pre-sterilized 18 x 150 mm crimp top tubes using a modification of Hungate anaerobic method as described by Bryant (1972). The tubes were supplemented (in triplicate) with small volumes (0.1 and 0.16 mL first and second experiments, respectively) of stock solutions of thymol or thymol-β-D-glucopyranoside (prepared in 10% ethanol) to achieve a final concentration of 1 mM. In each experiment, triplicate sets of tubes were also supplemented with the same volumes of 10% ethanol for use as controls. The tubes were inoculated with (0.2% vol/vol) *C. coli* in the first experiment or *C. jejuni* in the second experiment and the respective co-cultures were also inoculated with *P. distasonis* (0.2% vol/vol). Tubes were closed and incubated upright without agitation at 39°C. Fluids were collected at intervals and analyzed colorimetrically for determination of ammonia (108) and bacteriologically for viable cell count enumeration of *Campylobacter* as routinely done in this laboratory (109, 110). Briefly, samples were serially diluted (10-fold) in 0.1 M sodium phosphate buffer (pH 7.34) and spread-plated on Campy Cefex agar. Inoculated Campy Cefex agar

plates were incubated at 42ºC under a microaerophillic (CO_2:H_2:N_2) gas composition. Colonies were counted after 48-h incubation at 42°C. Gas chromatography analysis for free thymol (102) and pH measurements were made on remaining volumes of culture fluids from the pure and co-cultures of *C. jejuni* using.

Tests of thymol and thymol-β-D-glucopyranoside against *Campylobacter* culture with mixed populations of porcine, bovine or avian gastrointestinal tract microbes

To test the effect of thymol and thymol-β-D-glucopyranoside within mixed populations of gut bacteria, *C. coli* or *C jejuni* were cultured at 39°C under N_2 in anaerobic Bolton broth inoculated with bovine or porcine feces, obtained fresh from a mature cow or sow, and with freshly collected crop or cecal contents obtained by necropsy from a market-aged broiler. Fecal, crop and cecal contents were inoculated into Boltons broth at 0.2 g per 100 mL and *Campylobacter* were inoculated at 0.2% vol/vol. In mixed culture experiments, test compounds were added in small volumes (0.1 mL) of concentrated stock solutions of thymol or thymol-β-D-glucopyranoside (each dissolved in a 62.5% ethanol solution) to achieve a 1 mM final concentration. Equal volumes of water or 62.5% ethanol were similarly added to control incubations. Fluid samples were collected from each of the mixed cultures at intervals during incubation for colorimetric determination of ammonia and bacteriological enumeration of Campy-Cefex agar for enumeration of *Campylobacter* as described earlier. Portions of fluid samples from the mixed porcine and bovine fecal incubations were also analyzed for pH and concentrations of volatile fatty acid by gas chromatography using the method of (111)

and representative samples from mixed cultures of bovine microbes were also subjected to GC analysis for determination of free thymol as described earlier.

Tests of orally administered thymol and thymol-β-D-glucopyranoside against crop and cecal *Campylobacter* and generic *Escherichia coli* following a short-term preharvest intervention

Twenty commercially-reared market-aged broilers were acquired from Sanderson Farms (College Station, TX) early morning (approximately 09:00) and transported within 50 minutes to the Southern Plains Agricultural Research Center facilities. Over the course of the next 30 minutes, eighteen of the broilers were allocated to their respective treatments (6 per treatment) via placement to randomly selected individual layer cages fitted with fresh floor paper to obtain fresh fecal droppings for cultural determination of pre-treatment *Campylobacter* and *E. coli* status. Birds treatment groups were orally gavaged that evening at 20:00 and again at 24:00 with 3-mL volumes of 20% ethanol solution or 25mM thymol or thymol-β-D-glucopyranoside (each prepared in 20% ethanol), respectively. After the last treatment, the broilers were moved to a common floor pen (2.4 m x 3.6 m) bedded with fresh pine shavings. *Ad libitum* access to fresh water was provided in both rearing environments. The next morning at 09:00, each broiler was killed by cervical dislocation and necropsied within 1 h of harvest for collection of crop and cecal contents. Fecal droppings and contents were serially diluted (10-fold) in 0.1 M sodium phosphate buffer (pH 7.34) and spread-plated on to Campy

Cefex agar and to *E. coli*/Coliform Petrifilm (3M Microbiology, St, Paul, Mn) for enumeration of *Campylobacter* and *E. coli* (109, 110).

Statistics

All incubations were performed in triplicate. Concentrations of ammonia, volatile fatty acids and $\log_{10}$ transformations of CFU mL^{-1} of *C. coli* and *C. jejuni* were tested for treatment effects at each sampling time by a general analysis of variance. For the *in vitro* studies, treatment means were compared to controls using a two-sided Dunnett's Multiple comparison procedure. For the *in vivo* animal study, multiple comparison of means were accomplished using a least significant differences were considered significant at $P \leq 0.05$. All analysis Software were performed using Statistix© 9 Analytical Software (Tallahassee, FL, USA).

Results

Viable cell counts of *C. coli* during pure culture in medium supplemented with 1 mM thymol were reduced from those of non-treated control cultures after 24 h of incubation ($P< 0.05$) but only tended ($P= 0.0537$) to be lower after 48 h (Figure 1A). When grown in pure culture with 1mM thymol-β-D-glucopyranoside, viable counts of *C. coli* were increased nearly 2.0 $\log_{10}$ CFU mL^{-1} ($P< 0.05$) from those of controls after 24 h culture but were reduced, albeit not significantly, by 1.88 $\log_{10}$ CFU mL^{-1} from those of controls after 48-h culture. During co-culture with *P. distasonis*, viable counts of *C. coli* supplemented with 1 mM thymol were reduced from those of controls by 3.54 to 4.30 $\log_{10}$ CFU mL^{-1} after 6, 24, and 48-h culture, respectively (Figure 1B). Viable *C.*

coli counts did not differ from control counts after 24 h culture in medium supplemented with 1 mM added thymol-β-D-glucopyranoside but were reduced from control counts by 4.45 $\log_{10}$ CFU units mL^{-1} after 48-h culture with 1 mM thymol-β-D-glucopyranoside (Figure 1B).

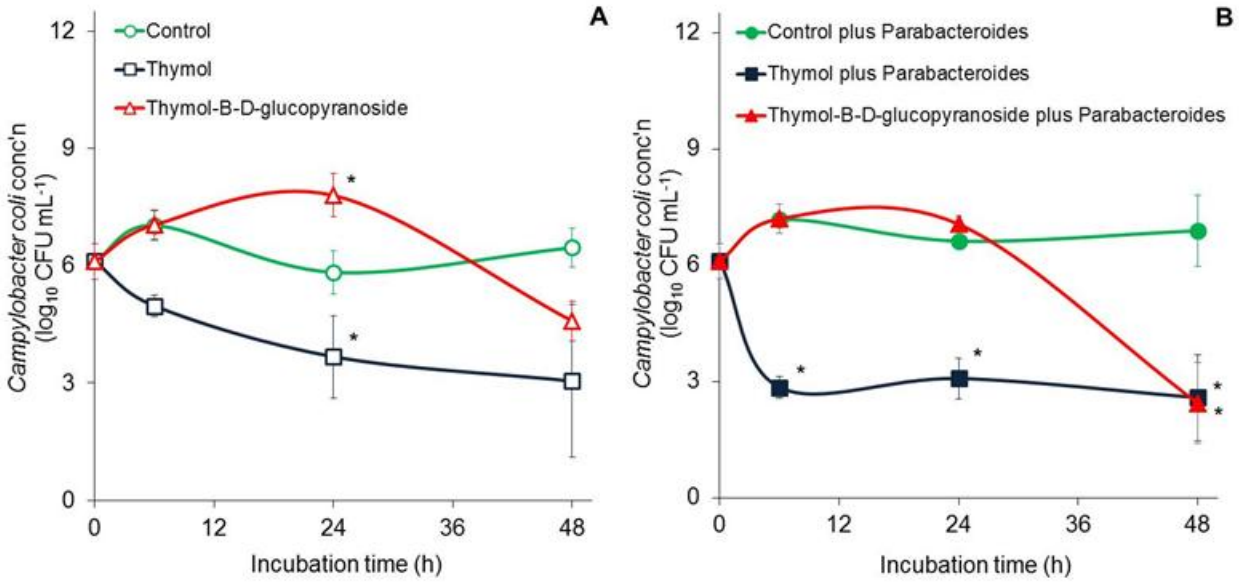

Figure 1. Viable cell counts during incubation of *Campylobacter coli* in pure culture (Figure A) or co-culture with *Parabacteroides distasonis* (Figure B) incubated anaerobically (N_2) at 39ºC in Bolton broth supplemented without (circles) or with either 1 mM thymol (squares) or thymol-β-D-glucopyranoside (triangles). Values are the mean ± SD from $n = 3$ cultures and asterisks indicate values differing from control values ($P < 0.05$).

For pure cultures of *C. jejuni*, counts were reduced ($P< 0.05$) by 1.88 and 4.80 $\log_{10}$ CFU mL^{-1} after 24 and 48-h incubation, incubation in Bolton broth supplemented

with 1mM thymol compared to controls but were not reduced in cultures treated with 1mM thymol-β-D-glucopyranoside (Figure 2A). Conversely, when co-cultured with a β-glycosidase expressing *P. distasonis*, counts of *C. jejuni* were reduced from those of controls by 6.43 and 6.87 $\log_{10}$ CFU mL^{-1} after 24 and 48-h, respectively, in cultures treated with 1 mM thymol (Figure 2B). As observed with *C. coli*, viable cell counts of *C. jejuni* were not reduced after 24 h, but were reduced after 48-h co-culture with *P. distasonis* in Bolton broth supplemented with 1 mM thymol-β-D-glucopyranoside, thus indicating that there was likely a lag in the hydrolysis of thymol from the glucose conjugate.

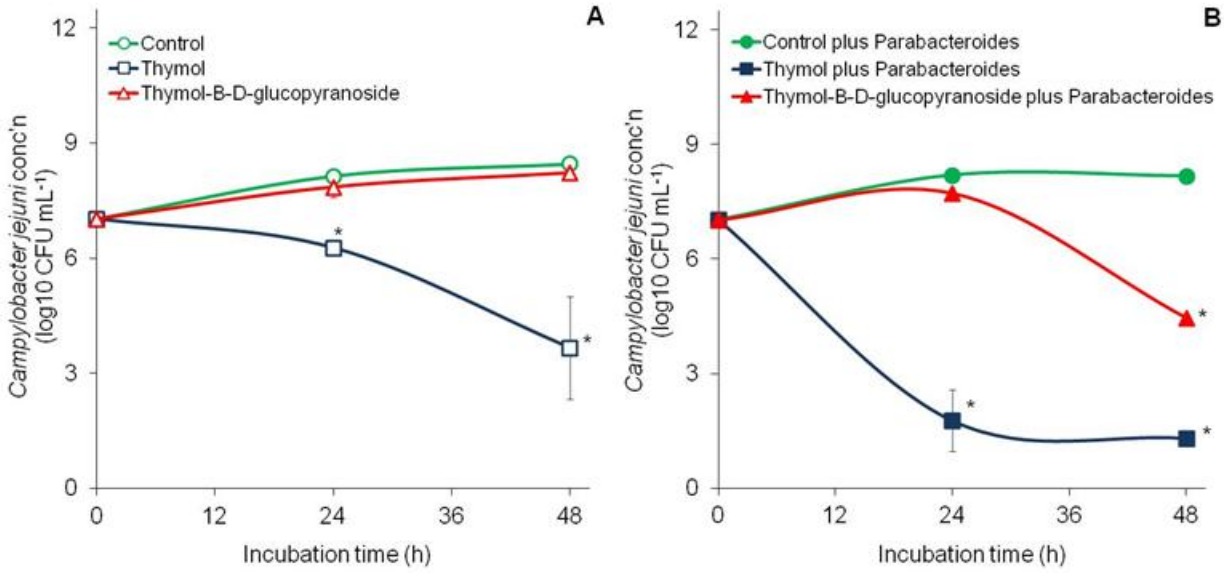

Figure 2. Viable cell counts during incubation of *Campylobacter jejuni* in pure culture (Figure A) or co-culture with *Parabacteroides distasonis* (Figure B) incubated anaerobically (N_2) at 39ºC in Bolton broth supplemented without (circles) or with either 1 mM thymol (squares) or thymol-β-D-glucopyranoside (triangles). Values are the mean ± SD from n = 3 cultures and asterisks indicate values differing from control values ($P < 0.05$).

In thymol-treated cultures, thymol concentrations measured in fluid samples collected after 6, 24 and 48-h incubation of pure culture of *C. jejuni* averaged (± SD) 0.52 ± 0.02, 0.48 ± 0.05 and 0.44 ± 0.01 μmol mL^{-1} and did not differ from each other ($P > 0.05$). However, these concentrations were decreased ($P < 0.05$) by approximately 48 to 56% from the initial 1 mM added thymol. Similarly, thymol concentrations averaged 0.58 ± 0.06, 0.64 ± 0.08 and 0.50 ± 0.04 μmol mL^{-1} in fluid samples collected after 6, 24 and 48 h incubation of co-cultures of *C. jejuni* and *P. distasonis* and were decreased ($P < 0.05$) by 36 to 50% from the initial 1 mM added thymol concentration. In co-cultures treated with 1 mM thymol-β-D-glucopyranoside, thymol accumulations were much lower even by 48-h of incubation (0.37 ± 0.02 μmol mL^{-1}) and never exceeded 0.2 μmol mL^{-1} in pure cultures treated with 1 mM thymol-β-D-glucopyranoside. Ammonia accumulations measured after 24 and 48 h incubation of *C. coli* were lower ($P < 0.05$) than controls during pure culture, but accumulations varied considerably and consequently were not significantly different from untreated controls during co-culture with *P. distasonis* when supplemented 1 mM thymol or 1mM thymol-β-D-glucopyranoside (Figure 3A and B). An effect of 1 mM thymol supplementation on ammonia accumulations was not observed after 6 h pure culture of *C. coli*, however, which probably reflects slow or lag phase growth during the early incubation period of this study.

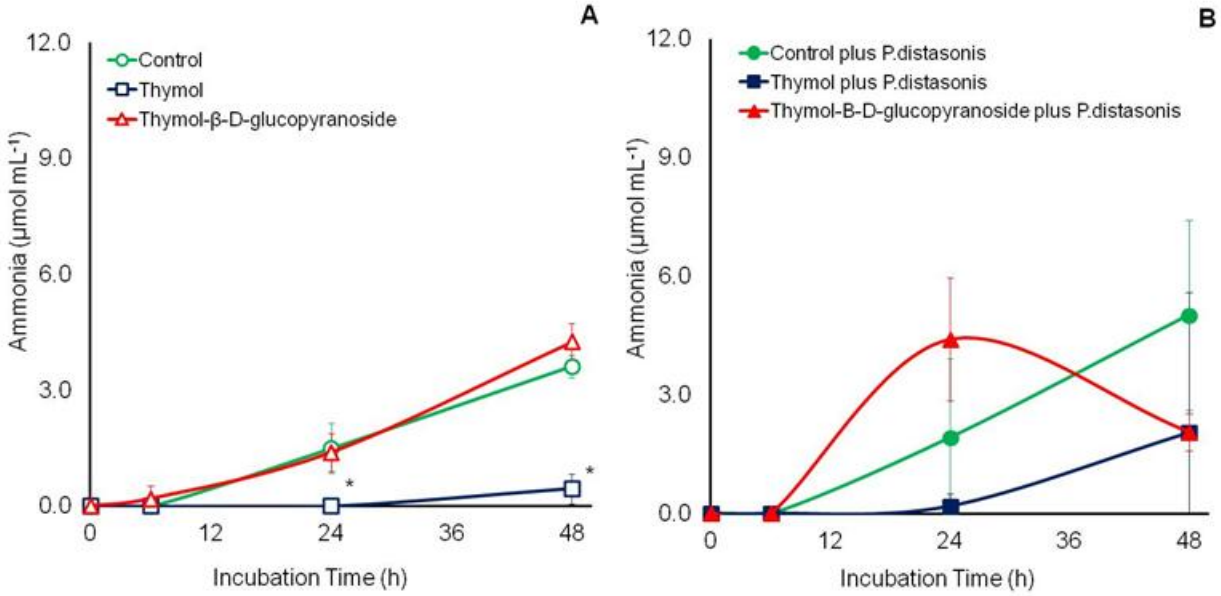

Figure 3. Accumulation of ammonia during incubation of *Campylobacter coli* in pure culture (Figure A) or co-culture with *Parabacteroides distasonis* (Figure B) incubated anaerobically (N_2) at 39ºC in Bolton broth supplemented without (circles) or with either 1 mM thymol (squares) or thymol-β-D-glucopyranoside (triangles). Values are the mean ± SD from $n = 3$ cultures and asterisks indicate values differing from control values ($P < 0.05$).

Ammonia accumulations measured after 24 and 48-h incubation of *C. jejuni* were lower ($P < 0.05$) than controls during pure as well as during co-culture with *P. distasonis* when supplemented with 1 mM thymol but not 1 mM thymol-β-D-glucopyranoside (Figure 4A and B). In the case of *C. jejuni*, pH measurements made at the end of the 48-h incubation were unaffected by treatment during pure culture ($P = 0.1679$) and averaged 7.40 ± 0.19. An effect of treatment was observed ($P = 0.0009$) on pH during

co-culture, however, with the pH measured after 48-h co-culture with thymol (7.47 ± 0.03) being higher ($P < 0.05$) than that measured in controls (7.22 ± 0.02). The pH in co-cultures treated with thymol-β-D-glucopyranoside (7.23 ± 0.07) did not differ from controls. Measurements of pH were not made during pure or co-culture of *C. coli*.

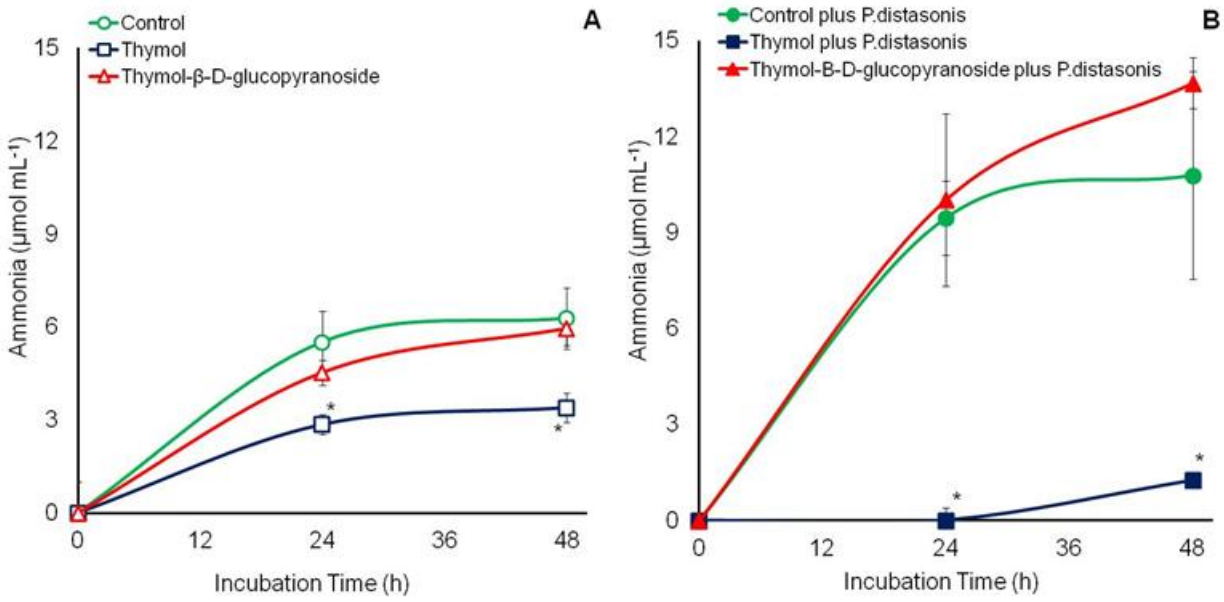

Figure 4. Accumulation of ammonia during incubation of *Campylobacter jejuni* in pure culture (Figure A) or co-culture with *Parabacteroides distasonis* (Figure B) incubated anaerobically (N_2) at 39ºC in Bolton broth supplemented without (circles) or with either 1 mM thymol (squares) or thymol-β-D-glucopyranoside (triangles). Values are the mean ± SD from $n = 3$ cultures and asterisks indicate values differing from control values ($P < 0.05$).

When grown in mixed culture, the freshly collected populations of porcine (Figure 5A) or bovine fecal microbes (Figure 5B), main effects of treatment were observed after 6 ($P \leq 0.0016$) and 24 h ($P \leq 0.0035$) of culture. For mixed porcine and bovine population treated with 1 mM thymol, viable counts of *C. coli* and *C. jejuni* were reduced ($P \leq 0.05$) from those of control by 3.11 and 4.74 $\log_{10}$ CFU mL^{-1}, respectively, after 6 h incubation and were reduced ($P < 0.05$) to 3.25 and 2.50 $\log_{10}$ CFU mL^{-1}, respectively, after 24 h incubation. For the mixed porcine populations treated with 1 mM thymol-β-D-glucopyranoside, *C. coli* counts were reduced ($P < 0.05$) from those of controls by 2.02 and 3.26 $\log_{10}$ CFU mL^{-1} after 6 and 24 h incubation, respectively. In the mixed bovine cultures treated with 1 mM thymol-β-D-glucopyranoside, *C. jejuni* counts did not differ ($P > 0.05$) from the control counts at the 6 h sampling time but were reduced ($P < 0.05$) from the controls by 2.40 $\log_{10}$ CFU mL^{-1}after 24 h of incubation.

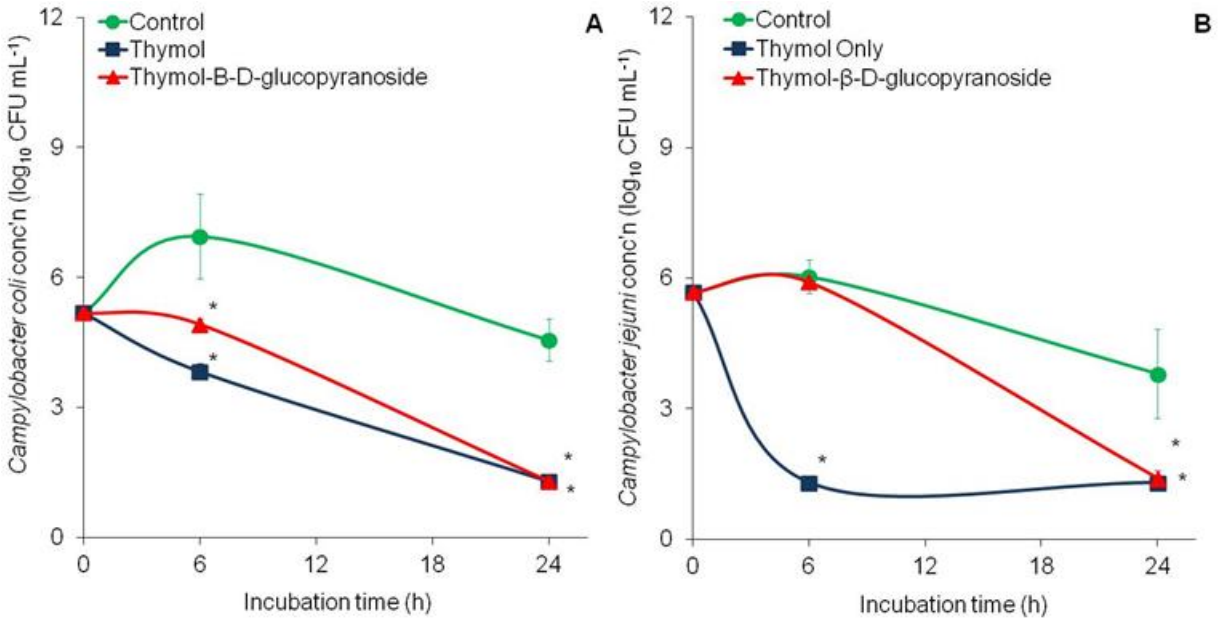

Figure 5. Viable cell counts of *Camylobacter coli* (Figure A) or *Campylobacter jejuni* (Figure B) during mixed culture of porcine or bovine gut microbes, respectively, incubated anaerobically (N_2) at 39ºC in Bolton broth supplemented without (circles) or with either 1 mM thymol (squares) or thymol-β-D-glucopyranoside (triangles). Values are the mean ± SD from n = 3 cultures and asterisks indicate values differing from control values ($P < 0.05$).

A main effect of thymol or thymol-β-D-glucopyranoside was not observed on ammonia accumulation by 6 h ($P = 0.4372$) mixed culture of porcine and bovine fecal microbe but an effect ($P < 0.0001$) was observed after 24 h culture, with accumulations in cultures treated with 1mM thymol or thymol-β-D-glucopyranoside being lower ($P < 0.05$) than those of untreated controls (Figures 6A and B).

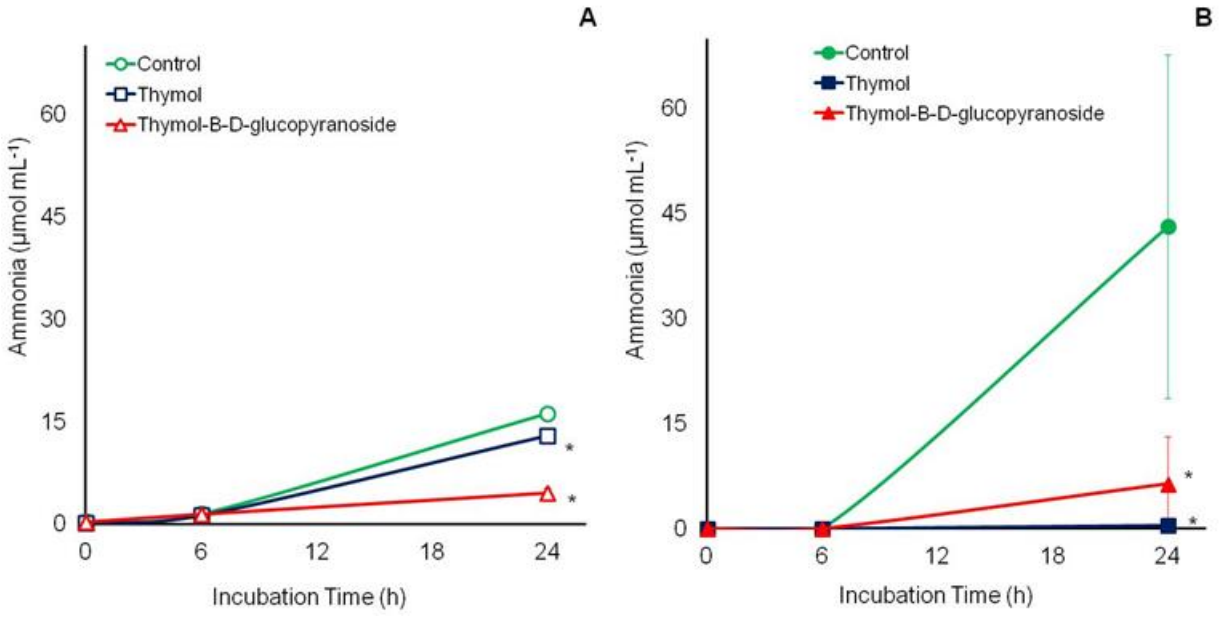

Figure 6. Accumulation of ammonia during mixed culture of *Campylobacter coli* or *Campylobacter jejuni* with porcine (Figure A) or bovine gut microbes (Figure B), respectively. Cultures were incubated anaerobically (N_2) at 39ºC in Bolton broth supplemented without (circles) or with either 1 mM thymol (squares) or or thymol-β-D-glucopyranoside (triangles). Values are the mean ± SD from $n = 3$ cultures and asterisks indicate values differing from control values ($P < 0.05$).

A main effect of treatment ($P \leq 0.0142$) was observed on accumulations of the fermentation acids acetate and propionate in the mixed cultures of porcine (7A and B) fecal microbes incubated 24 h, with accumulations by cultures treated with 1 mM thymol-β-D-glucopyranoside being reduced ($P < 0.05$) from those of the controls. Effects of treatment on accumulations of butyrate, valerate and branched chain fatty

acids isobutyrate and isovalerate were also observed ($P \leq 0.0192$), with concentrations being decreased from those of controls in the mixed cultures of porcine fecal microbes supplemented with 1 mM thymol-β-D-glucopyranoside (Figures 7A and B).

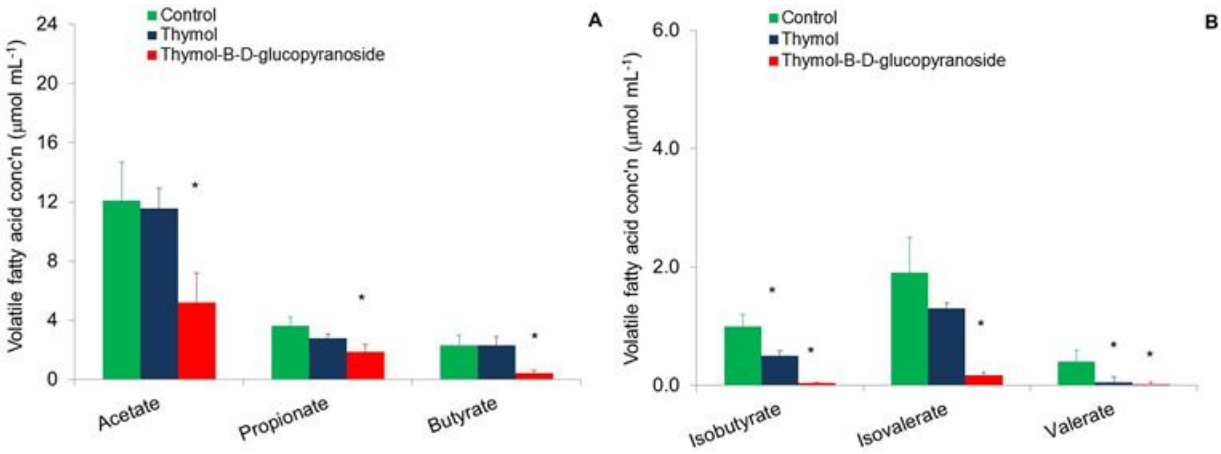

Figure 7. Accumulation of fermentation acids during mixed culture of *Campylobacter coli* with porcine gut microbes. Cultures were incubated anaerobically (N_2) at 39ºC in Bolton broth supplemented without (green) or with either 1 mM thymol (blue) or thymol-β-D-glucopyranoside (red). Values are the mean ± SD from $n = 3$ cultures and asterisks indicate values differing from control values ($P < 0.05$).

Accumulations of the branched chain volatile fatty acids isobutyrate and valerate by mixed porcine cultures treated with 1 mM thymol were lower ($P < 0.05$) than those of controls but accumulations of the other volatile fatty acids did not differ. A main effect of treatment was observed ($P \leq 0.0226$) on accumulations of acetate and propionate during mixed culture of *C. jejuni* with bovine fecal microbes (Figures 8A and B) with

accumulations in cultures treated with 1 mM thymol-β-D-glucopyranoside being lower than accumulations in non-treated controls. Treatment effects were not observed ($P \geq$ 0.3564) on accumulations of any of the other volatile fatty acids in the mixed cultures of bovine fecal microbes. A main effect of treatment was observed on the final pH during (P = 0.0014 and 0.0046, respectively) mixed culture of the porcine and bovine fecal microbes. For the mixed porcine cultures, the final pH was higher ($P < 0.05$) in controls (6.90 ± 0.03) than in cultures treated with 1 mM thymol or 1 mM thymol-β-D-glucopyranoside (6.75 ± 0.02 and 6.59 ± 0.09, respectively). In the case of the mixed bovine cultures, the final pH was higher ($P <$ 0.05) in the thymol-treated (7.34 ± 0.31) cultures than in controls (6.62 ± 0.07), but the pH in cultures treated with thymol-β-D-glucopyranoside (6.67 ± 0.11) did not differ from the controls.

Free thymol accumulations in fluid samples collected from mixed cultures of bovine fecal microbes incubated with the addition of 1 mM free thymol were 0.93 ± 0.36 and 0.77 ± 0.21 μmol mL^{-1} at 6 and 24 h and were not different ($P > 0.05$) than the amount added. Free thymol accumulations in fluid samples collected from mixed bovine fecal cultures treated with the addition of 1 mM thymol-β-D-glucopyranoside were 0.14 ± 0.02 and 1.28 ± 0.49 μmol mL^{-1} at 6 and 24 h, respectively

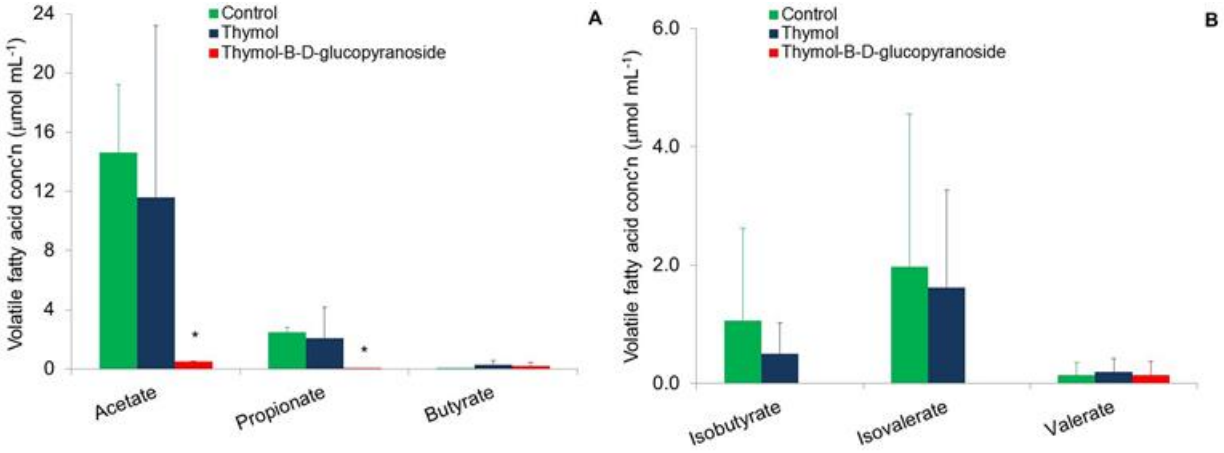

Figure 8. Accumulation of fermentation acids during mixed culture of *Campylobacter jejuni* with bovine gut microbes. Cultures were incubated anaerobically (N_2) at 39ºC in Bolton broth supplemented without (green) or with either 1 mM thymol (blue) or thymol-β-D-glucopyranoside (red). Values are the mean ± SD from $n = 3$ cultures and asterisks indicate values differing from control values ($P < 0.05$).

When cultured with mixed populations of freshly collected avian crop (Figure 9A) or cecal microbes (Figure 9B), bacterial activity of both 1 mM thymol and 1 mM thymol-β-D-glucopyranoside against *C. jejuni* was again observed, but only after 24 h incubation. The magnitude of the bactericidal effect ranged from 1.10 to 2.32 $\log_{10}$ CFU mL^{-1} for these two compounds, which appears to be considerably less dramatic in the broiler crop and cecal populations than that observed with porcine or bovine fecal populations.

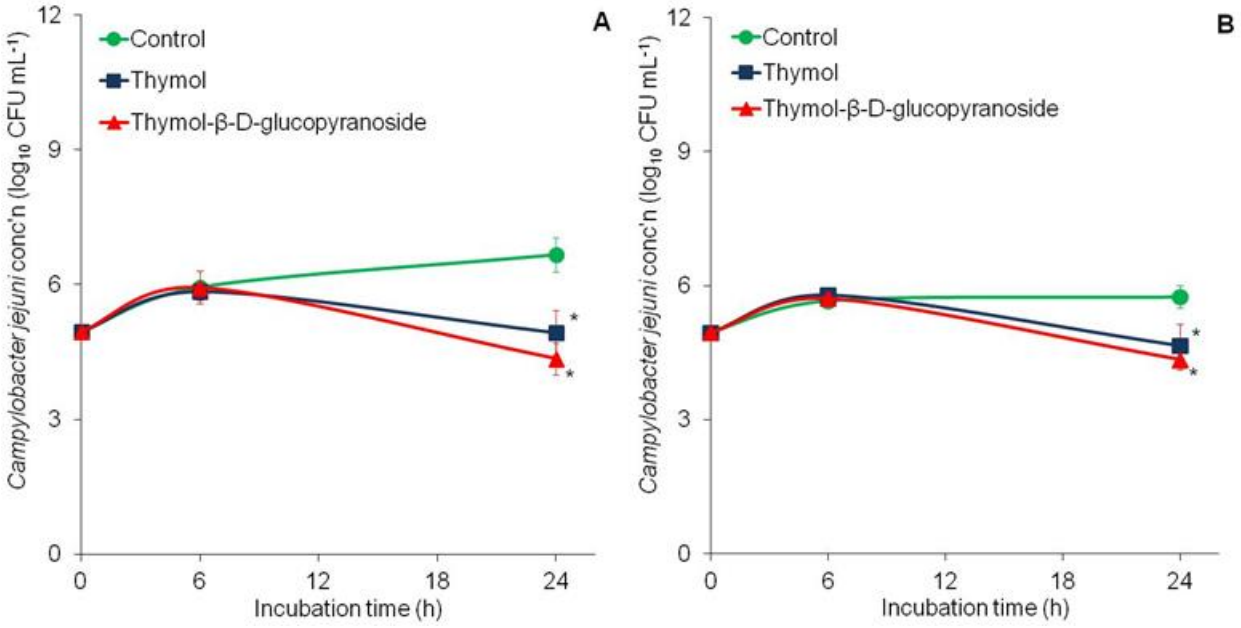

Figure 9. Viable cell counts of *Campylobacter jejuni* during mixed culture with avian gut microbes originating from the crop (Figure A) or ceca (Figure B). Cultures were incubated anaerobically (N_2) at 39ºC in Bolton broth supplemented without (circles) or with either 1 mM thymol (squares) or thymol-β-D-glucopyranoside (triangles). Values are the mean ± SD from $n = 3$ cultures and asterisks indicate values differing from control values ($P < 0.05$).

A main effect of treatment was not observed on the ammonia accumulations after 6 or 24 h culture of *C. jejuni* (Figures 10A and B) and mixed populations of avian crop bacteria ($P \geq 0.5403$). There was not a main effect of treatment observed after 6 h culture of mixed populations of avian cecal bacteria ($P = 0.0341$) on ammonia accumulations nor after 24 h mixed culture of avian cecal microbes, with ammonia concentrations being higher ($P < 0.05$) for cultures treated with 1 mM thymol or 1 mM thymol-β-D-glucopyranoside than in untreated control cultures (Figure 10B).

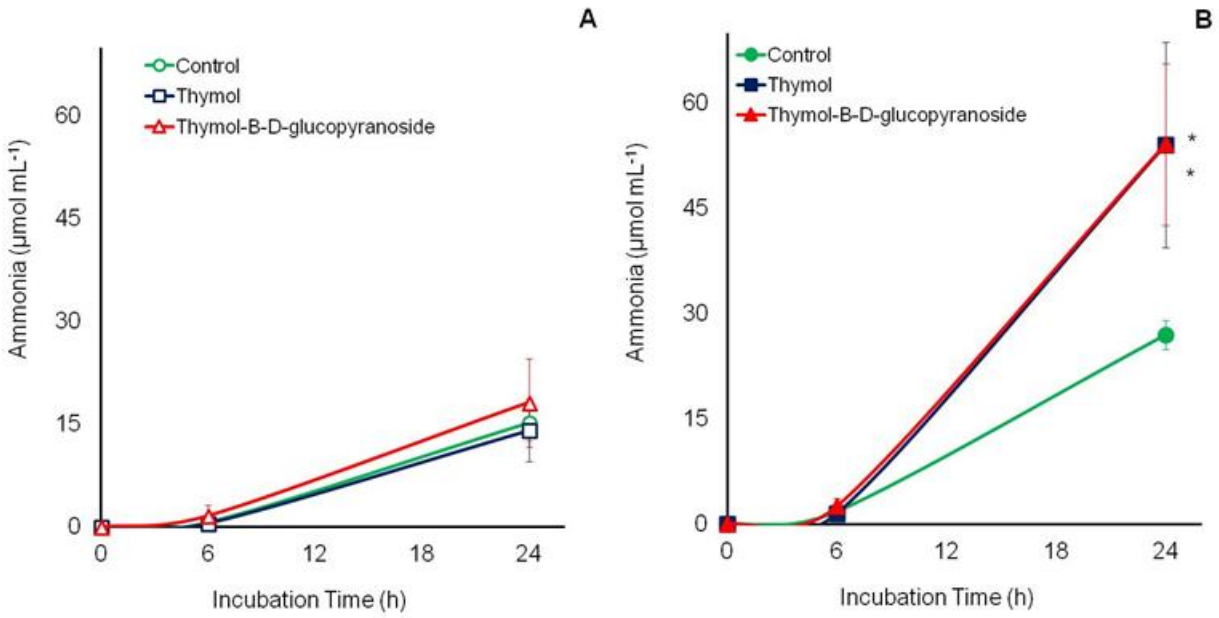

Figure 10. Accumulation of ammonia during mixed culture of *Campylobacter jejuni* with avian gut microbes originating from the crop (Figure A) or ceca (Figure B). Cultures were incubated anaerobically (N_2) at 39ºC in Bolton broth supplemented without (circles) or with either 1 mM thymol (squares) or thymol-β-D-glucopyranoside (triangles). Values are the mean ± SD from n = 3 cultures and asterisks indicate values differing from control values ($P < 0.05$).

A main effect of treatment was observed on concentrations of viable *Campylobacter* ($P = 0.0233$) recovered from crop contents collected at necropsy. Counts were 1.2 $\log_{10}$ CFU mL^{-1} units lower in the contents from birds treated with thymol-β-D-glucopyranoside than in birds treated with thymol (Figure 11). Recovery of viable *Campylobacter* in cecal contents was unaffected (P =0.1494) by treatment, recovery of viable *E. coli* spp. from crop or cecal contents was also unaffected (P = 0.8997 and

0.5601, respectively) (Figure 11). Ammonia accumulations in the crop and cecal contents were unaffected by treatment (P = 0.4994 and 0.2855, respectively) and averaged 0.06 ± 0.10 µmol NH_3 g^{-1} crop content and 0.77 ± 0.34 µmol NH_3 g^{-1} cecal content.

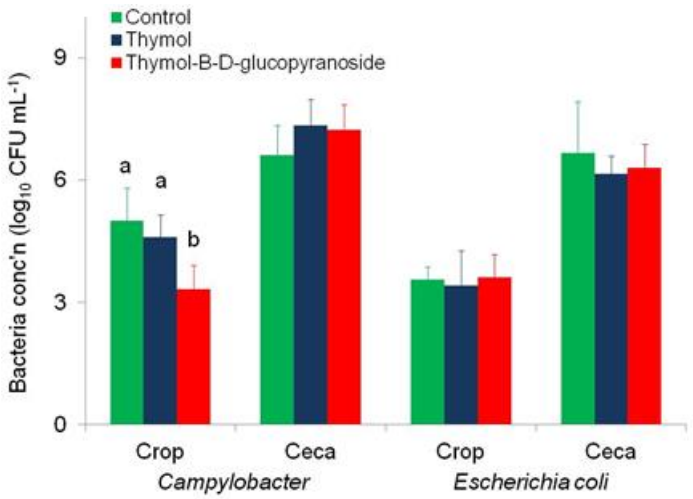

Figure 11. Recovery of viable *Campylobacter* and *Escherichia coli* spp. from crop and cecal contents obtained upon necropsy of market aged broilers (n = 6 per treatment) twice-treated (approximately 11 and 15 hr previously) via oral gavage with 3 ml volumes of 25 mM thymol (blue) or thymol-β-D-glucopyranoside (red). Control birds (n = 6) were similarly treated with equal volumes of 20% ethanol (green). Values are the mean ± SD and where indicated with unlike lower case letters, values differ (P < 0.05) based on an LSD comparison of means.

Discussion

Results from the present study confirm the results from the earlier work of Anderson et al.(1) showing that 1 mM thymol markedly reduced the survivability of *C. coli* and *C. jejuni* both in pure and mixed culture with porcine and bovine gut microbes. Moreover, the 1 mM thymol treatment reduced the survivability of *C. jejuni* during mixed culture with avian crop and cecal microbes, although not to the same extent as in the bovine or porcine cultures. Numerous other studies have found that thymol is bactericidal to important foodborne pathogens with a minimum bactericidal concentrations reported to be 0.66, 1.10 and 1.55 mM for *Escherichia coli* K88, *E. coli* O157:H7 and *Salmonella enterica* serovar Typhimurium DT 104, respectively (112). Total anaerobes from the pig intestine were less susceptible to 1.72 mM thymol (26, 113). Mechanistically, thymol is thought to exert its bactericidal effect via disruption of the bacterial cell wall (24), although the ability of thymol to inhibit deaminase activity has been proposed as a potential mechanism limiting the growth of assacharolytic *Campylobacter* (1). With respect to the latter mechanism, accumulations of ammonia were decreased in the present study by 1 mM thymol during pure culture of *C. coli* and *C. jejuni* as well as during co-culture with *P. distasonis*, albeit ammonia accumulations during co-culture of *C. coli* were not decreased significantly, likely because of the high amount of variability in ammonia measurements in these cultures.

Despite the clearly evident bactericidal activity of thymol, its use as a preharvest feed additive to reduce the colonization of foodborne pathogens in the lower gut is likely limited because it is rapidly and extensively absorbed or degraded in the proximal

alimentary tract (101). Consequently, some researchers have proposed that encapsulation or other protection technologies will likely be needed to deliver effective concentrations of thymol to the lower gut to achieve *in vivo* reductions of *Campylobacter*. Whereas the use of natural and synthetic glycosidic conjugates to deliver pharmaceuticals to the colon has been used previously for a variety of other compounds (114), the use of β-glycosides to deliver thymol to the lower gut has not previously been investigated. Conceptually, if constructed properly intact β-glycosides should be resistant to absorption. In that regard, recent evidence has shown that the intact thymol-β-D-glucopyranoside glycoside is much more resistant to absorption, being absorbed across everted porcine jejunal segments at 1/3 the rate of free thymol (102).

Additionally, in monogastric animals the intact glycosides should be resistant to hydrolysis within the proximal alimentary tract, because higher animals are limited in their ability to produce β-glycoside-hydrolyzing enzymes. This activity is expressed in momogastrics by their populations of gut microbes colonizing the lower tract. Within pigs, for example, β-glycosidase activity is low or absent in the stomach and proximal small intestine. Activity does not become appreciable until the distal small intestine, cecum or large intestine where populations of competent hydrolyzing bacteria are established (115). Competent β-glycosidase expressing bacteria include certain species belonging to Bacteroides, Bifobacteria, Clostridia, Enterobacteria, Enterococci and lactobacilli (116). The *P. distasonis* used in co-culture studies is also known to express β-glycosidase activity and is a normal inhabitant of gut habitats (117).

In the present study, the 1 mM thymol-β-D-glucopyranoside treatment was inhibitory to *C. coli* and *C. jejuni* but this activity was significant only when co-cultured with *P. distasonis* or when cultured with mixed populations of gut bacteria. These results provide evidence that the bactericidal activity of thymol-β-D-glucopyranoside was dependent on the presence of microbial populations possessing enzymatic activity capable of hydrolyzing the glycosidic bond. Conversely, the lack of an effect of thymol-β-D-glucopyranoside on the recovery of viable *C. coli* and *C. jejuni* during pure culture is not unexpected considering there is no evidence that these two *Campylobacter* species express β-glycosidase (118). Furthermore, only very low amounts of free thymol (< 0.20 mM) were measured in pure cultures of *C. jejuni* treated with thymol-β-D-glucopyranoside and this may likely have been added as a contaminant of the synthesized thymol-β-D-glucopyranoside.

Similarly, ammonia accumulations were not significantly affected by thymol-β-D-glucopyranoside during pure culture of *C. coli* and *C. jejuni* which suggests that appreciable amounts of free thymol were not made available to inhibit amino acid deamination. Significant effects of thymol-β-D-glucopyranoside were not observed on ammonia accumulation during co-culture either and this is contrary to what would be expected since the β-glycosidase activity of *P. distasonis* should liberate free thymol. It is possible, though, that the considerable contribution of *P. distasonis* to ammonia production may have masked an effect of any thymol potentially liberated during co-culture. Moreover, the bactericidal effect of the 1 mM thymol-β-D-glucopyranoside against *C. coli* and *C. jejuni* during co-culture was not apparent until the 48-h sampling

period which suggests that the liberation of free thymol from the glucose conjugate occurred slowly and late during culture. The low recovery of free thymol (0.37 ± 0.02 mM) from incubation fluids collected after 48 h co-culture of *C. jejuni* and *P. distasonis* provide further evidence the hydrolysis occurred slowly. Effects of thymol and thymol-β-D-glucopyranoside treatment on ammonia production during mixed culture were dependent on the source of the mixed microbial population. For example, significant effects of 1mM thymol or 1 mM thymol-β-D-glucopyranoside treatment on ammonia accumulations were observed during culture of *C. coli* and *C. jejuni* with the respective porcine and bovine fecal populations. But again this inhibition did not occur until late during culture.

When measured in fluid samples collected from the mixed bovine cultures, appreciable free thymol did not accumulate in the cultures treated with 1 mM thymol-β-D-glucopyranoside until after 24 h. In contrast to that observed with the mixed populations of porcine and bovine microbes, ammonia accumulation by mixed populations of avian crop microbes were unaffected by treatment with 1 mM thymol or thymol-β-D-glucopyranoside and were increased in cultures of avian cecal microbes. The differential effects observed between the different populations are not readily explained but are likely to be population specific.

From a practical perspective, it is reasonable to suspect that thymol-β-D-glucopyranoside would be most suited for use as a feed additive in pigs because little hydrolysis of the glucose conjugate would be expected to occur within the pig stomach and proximal small intestine. Conversely, a major challenge to the use of thymol-β-D-

glucopyranoside or other β-glycosidic-linked conjugates in ruminant or poultry is the presence of competent β-glycoside hydrolyzing microbial populations in their rumen or crop. For example, when live broilers in this study were orally treated with thymol or thymol-β-D-glucopyranoside, only thymol-β-D-glucopyranoside was effective in reducing *Campylobacter* and only in the crop. These results suggest that thymol was indeed absorbed very rapidly, perhaps as early as in the crop. Additionally, the lack of a thymol-β-D-glucopyranoside effect in the ceca suggests that the β-glycoside was extensively hydrolyzed in the crop and the liberated thymol was subsequently absorbed during passage to the ceca. The doses administered to the broilers were designed, based on estimates of gut volume, to deliver between 1 to 3 mM thymol or thymol-β-D-glucopyranoside to the compartments and it is possible these doses may have been too low to allow sufficient passage of the compounds. For example, it is possible that higher doses may allow sufficient amounts to escape absorption or hydrolysis in the crop. Conversely, if additional encapsulation or the protective technologies could be used to deliver the intact glycoside to the lower gut, results from the *in vitro* study suggested that the compounds possibly could be active there.

Importantly, however, results from the *in vitro* incubations with mixed populations of bovine fecal microbes revealed that thymol-β-D-glucopyranoside treatment markedly inhibited accumulations of major fermentative acids, acetate and propionate. The finding indicates that the thymol-β-D-glucopyranoside was a potent inhibitor of fermentation which likely will restrict its potential use as a preharvest feed

additive. Possibly, it could be administered as a terminal strategy, one given in the animals' last meal or perhaps in the water upon arrival at the processing plant.

In summary, results from the present studies extend the findings of thymols' antimicrobial activity in addition to addressing the issues pertaining to absorption within the stomach and small intestine. These results demonstrated that thymol and thymol-β-D-glucopyranoside inhibited *C. coli* and *C. jejuni* during pure culture, mixed populations of bovine, porcine and avian gut microbes. Bactericidal activity of thymol-β-D-glucopyranoside against *C. coli* and *C, jejuni* was dependent on those mixed populations contributing β-glycosidase activity. Further studies are clearly needed to elucidate practical and economic issues pertaining to its use as a feed additive.

CHAPTER III

CONCLUSION

Thymol-β-D-glucopyranoside was found to be much more resistant to absorption across the small intestine epithelium than free thymol. This result indicates that the conjugate can resist absorption in the proximal alimentary and thus pass intact to the lower gut where it can be activated. In its conjugate form, thymol-β-D-glucopyranoside has little if any biological activity against *Campylobacter* or against ammonia accumulation. These results demonstrated that once activated (hydrolyzed) the conjugate thymol-β-D-glucopyranoside was effectively bactericidal against *C. coli* and *C. jejuni* and supported ammonia accumulation in mixed and pure cultures of bovine, porcine, and avian gut microbes.

The results from the pure and co-culture results also revealed the necessity of β-glycosidase to activate (hydrolyze) the conjugate to promote the bactericidal activity of thymol. Thymol-β-D-glucopyranosides' significant reduction of the fermentation amino acids suggests that the use of the conjugate as a feed additive would be anti-nutritional. This result indicates a need for strategic planning of when and how much to implement or use thymol-β-D-glucopyranoside as a feed additive. In order to not affect the animals' production, implementation or use in a terminal meal for delivery of thymol-β-D-glucopyranoside seems most logical. The results from the *in vivo* avian study reveal the possible need for additional technologies or a dosage adjustment to prevent pre-intestinal or pre-cecal hydrolysis of the conjugate and of course each animal will require a different strategy.

The development of a practical cost effective method to implement a pre-harvest control strategy could be instrumental in reducing the concentration and incidence of *Campylobacter* spp. and other microbial pathogens in food-producing animals.

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additives towards selected pathogenic and beneficial gut bacteria. J Appl Microbiol **100:**296-305.

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APPENDIX

SHARON V. R. EPPS

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EDUCATION

Texas A&M University, College Station, TX,
Masters Science: Toxicology, **August 2013** **2013**
Thesis: The Effect of Thymol-β-D-Glucopyranoside on The Reduction of
Campylobacter Species in Food-Producing Animals

San Diego State University, San Diego, CA
B.S., Biology/Minor, Africana Studies **2007**

AWARDS

Anaerobe Society of the Americas 11th Biennial Congress Young Investigators Research
Poster Competition 1st Place **July 29, 2012**

TEACHING EXPERIENCE

Texas A&M University, College Station, TX
Teaching Assistant-To Dr Ellis in Genetics 301, Dept. Biochemistry & Biophysics **2007-2009**
Lectures, teaching basic genetic laboratory skills, laboratory preparation, grading
of lab analyses, problem sets and quizzes, and help desk duties.

San Diego State University, San Diego, CA
Teaching Assistant-To Dr Estralita Martin in Biology 100, EOP Summer Program **2005**
Administering test, grading and supervising mandatory study sessions

RELATED EXPERIENCE

United States Department of Agriculture (USDA/ARS) Food and Safety Research Unit
Graduate Student/Biological Technician **August**
2011 – Present

Provide research assistance involving microbial investigation in food
safety, DNA extraction, PCR, Denaturing Degradient Gel
Electrophoresis (DDGE) gel imaging and bacterial investigation.

PRESENTATIONS:

ORAL

Epps, S.V. R.,(2012, March) Activation of Thymol-β-D-Glucopyranoside By Bacterial-Expressed β-Glycosidase. TAMU Student Research Competition, Texas A&M University College Station Texas

Epps, S.V.R.,(2010, April) Retained Bactericidal Activity of Diphenyliodonium Chloride Impregnated in Alginate Beads Following Exposure to Stomach Simulating pH Conditions VIBS, Toxicology Department Graduate Student Seminars

Epps, S.V. R., (2007, March) *Alphaβet Soup of Escherichia coli Glycerol Kinase ATP-IIA^{Glc} Allosteric Coupling.*2007 TAMU Student Research Competition Texas A&M University College Station Texas

Epps, S.V. R., (2005, August) *Isolation of P-Insulin from Momordica charantia* ***Linn. (Cucurbitaceae),*** *"The African Cucumber": A herb used for the Treatment of Diabetes Mellitus.* Oral presentation at the University California San Diego (UCSD) McNair Scholars Research Symposium

Epps, S.V. R.,(2005, March) *Isolation of P-Insulin from Momordica charantia* ***Linn. (Cucurbitaceae),*** *"The African Cucumber": A herb used for the Treatment of Diabetes Mellitus.* Oral presentation at SDSU Cal-State competition

POSTERS

Epps, V.R., S., (2012, July) Activation of Thymol-β-D-Glucopyranoside By Bacterial-Expressed β-Glycosidase, Anaerobe Society of the Americas 2012 Young Investigator Competition 11th Biennial Congress, San Francisco, CA.

Epps, S.V. R., (2007, November) *Alphaβet Soup of Escherichia coli Glycerol Kinase ATP-IIA^{Glc} Allosteric Coupling.* Poster presented at 2007 ABRCMS conference in Dallas Texas

Epps, S.V. R., (2005, September) *Isolation of P-Insulin from Momordica charantia* ***Linn.***

(Cucurbitaceae), *"The African Cucumber": A herb used for the Treatment of Diabetes Mellitus.* Poster presented at 2005 SACNAS conference in Denver Colorado

PUBLICATIONS AND PAPERS

Ex vivo absorption of Thymol and Thymol-β-D-glucopyranoside in Piglet Everted Jejunal Segments. Petrujkic, B.T., Sedej, I., Bier, R.C. Anderson, R. C., Harvey, R.B., Epps, S.V. R., Stipanovic, R.D., Krueger, N.A., Nisbet, D.J. 2013 *J. Agric. Food Chem.* 61: 3757-3762 **2013**

MEMBERSHIPS

- Black Graduate Student Association Texas A&M University September 2006- Present
- Women in Science and Engineering (WISE) February 2007- Present
- Sigma XI Associate member May 3, 2005-Present
- American Society of Biochemistry & Molecular Biology (ASBMB) May 2005-Present
- AAAS February 2005-Present
- Louis Stokes AMP scholarship recipient, Spring 2005(SDSU)
- Ronald E. McNair Scholar 2004 San Diego State University
- MIRT Scholar Summer 2004 San Diego State University
- Black Student Science Organization President, (BSSO) Fall 2005-Spring 2006

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